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Exhibit I

Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity

Heather Knight,[1,*], Anthony J. Trewavas[2] and Marc R. Knight[1]

[1]*Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom and*

[2]*Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, UK*

Summary

Changes in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in response to mannitol (drought) and salt treatments were detected *in vivo* in intact whole *Arabidopsis* seedlings. Transient elevations of $[Ca^{2+}]_{cyt}$ to around 1.5 μM were observed, and these were substantially inhibited by pre-treatment with the calcium-channel blocker lanthanum and to a lesser extent, the calcium-chelator EGTA. The expression of three genes, *p5cs*, which encodes Δ[1]-pyrroline-5-carboxylate synthetase (P5CS), the first enzyme of the proline biosynthesis pathway, *rab18* and *lti78* which both encode proteins of unknown function, was induced by mannitol and salt treatments. The induction of all three genes by mannitol was inhibited by pretreatment with lanthanum. Salt-induced *p5cs*, but not *rab18* and *lti78*, expression was also inhibited by lanthanum. Induction of *p5cs* by mannitol was also inhibited by the calcium channel-blockers gadolinium and verapamil and the calcium chelator EGTA, further suggesting the involvement of calcium signalling in this response. Mannitol induced greater levels of *p5cs* gene expression than an isoosmolar concentration of salt, at both relatively high and low concentrations. However, calcium transients were of a similar magnitude and duration in response to both mannitol and isoosmolar concentrations of salt, suggesting that a factor other than calcium is involved in the discrimination between drought and salinity signals in *Arabidopsis*. In order to gauge the involvement of the vacuole as an intracellular calcium store in the response of *Arabidopsis* to mannitol, $[Ca^{2+}]_{cyt}$ was measured at the microdomain adjacent to the vacuolar membrane. The results obtained were consistent with a significant calcium release from the vacuole contributing to the overall mannitol-induced $[Ca^{2+}]_{cyt}$ response. Data obtained by using inhibitors of inositol signalling suggested that this release was occurring through IP_3-dependent calcium channels.

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*For correspondence (fax +44 1865 275023; e-mail heather.knight@plants.ox.ac.uk).

Introduction

Salinity has a considerable effect on world agriculture, with as much as half of the irrigated areas of land being affected by high salinity (Läuchli, 1991), accounting for 6% of the world's land surface (Flowers and Yeo, 1995). Salinity causes a number of detrimental effects; the resultant high osmotic pressure causes plants difficulty in absorbing water, and salt ions interfere with the uptake of nutrients as well as directly inhibiting cellular metabolism (Pasternak, 1987; Werner and Finkelstein, 1995). Drought has similar effects on osmotic potential and results in a number of modifications including closure of stomata to prevent further water loss (Davies *et al.*, 1981) and the accumulation of both proline (Savouré *et al.*, 1995) and other compatible osmoprotectants (Bartels and Nelson, 1994).

Proline appears to play a protective role in plant tolerance of salt and drought stresses, and accumulates after exposure to these stresses (Savouré *et al.*, 1995). The *At-P5CS* gene of *Arabidopsis* encodes the enzyme Δ[1]-pyrroline-5-carboxylate synthetase (P5CS), the first enzyme of the proline biosynthetic pathway and is strongly regulated by drought and salinity stress (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995). This biosynthetic pathway takes place in the cytoplasm, with P5CS converting glutamate into Δ[1]-pyrroline-5-carboxylate in a two-step reaction (Delauney and Verma, 1993). Δ[1]-pyrroline-5-carboxylate is then converted to proline by Δ[1]-pyrroline-5-carboxylate reductase. P5CS is thought to be the rate-limiting step in proline biosynthesis (Delauney and Verma, 1993). Overexpression of *At-P5CS* in transgenic plants increases osmotolerance by increasing proline levels, demonstrating that this enzyme has a key protective role in determining the survival of plants subjected to drought stress (Kishor *et al.*, 1995). RAB18 and LTI78 proteins both accumulate in response to drought and low temperature (Mäntylä *et al.*, 1995), both of these stresses involving dehydration. Therefore, although the roles of these proteins are not known, it has been suggested that they may mediate some of the responses to effects of drought-induced and low temperature-induced dehydration stress (Mäntylä *et al.*, 1995), probably in a protective capacity. Drought stress has been shown previously to cause increased expression of both the *lti78* gene (Nordin *et al.*, 1993) and the *rab18* gene (Lång *et al.*, 1994), *rab18* via an ABA-regulated signal transduction pathway (Lång and Palva 1992; Lång *et al.*, 1994) and *lti78* through an ABA-independent pathway (Nordin *et al.*, 1991).

Calcium signalling has been implicated in the transduction of drought- and salt-stress signals in plants and may

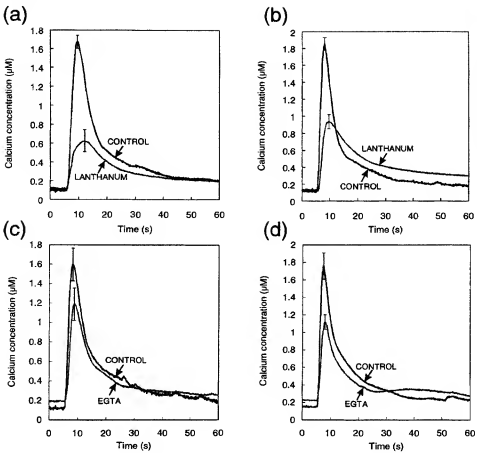

Figure 1. Mannitol and salt-induced $[Ca^{2+}]_{cyt}$ responses in *Arabidopsis* seedlings. Graphs show $[Ca^{2+}]_{cyt}$ response to
(a) 0.666 M mannitol,
(b) 0.333 M salt, both with (half-tone) and without (full-tone) lanthanum pretreatment,
(c) 0.666 M mannitol, and
(d) 0.333 M salt, both with (half-tone) and without (full-tone) EGTA pretreatment.
Salt or mannitol was added 5 sec after the beginning of the trace. Traces shown are averages of five individual measurements and error bars indicate standard error of the mean.

play a role in a number of responses to drought and changes in water potential (Davies *et al.*, 1981; Johansson *et al.*, 1996; Takahashi *et al.*, 1997). The discovery of drought- and high salt-induced expression of calcium-dependent protein kinases (Wimmers *et al.*, 1992; Urao *et al.*, 1994), hyperosmotic shock-induction of putative calcium-binding proteins (Ko and Lee, 1995; Pestacz and Erdei, 1996), and salt stress-induced expression of a putative Ca^{2+}-ATPase (Perez-Prat *et al.*, 1992) in plants and algae provides indirect evidence of the importance of calcium in these responses. The observation that salt stress causes elevations in intracellular calcium (Lynch *et al.*, 1989) in isolated maize root protoplasts constitutes more direct evidence. Apart from this very little is known about the early signalling events leading to drought- and salt-induced gene expression in plants and therefore we investigated this in *Arabidopsis*.

Results

In order to investigate the role of intracellular calcium in drought and salination signalling in *Arabidopsis*, we measured the *in vivo* changes in $[Ca^{2+}]_{cyt}$ which occur in response to these two stimuli (Figure 1). Luminometry of *Arabidopsis* plants expressing cytosolic aequorin (Knight *et al.*, 1991) revealed a similar transient increase in $[Ca^{2+}]_{cyt}$ in response to 0.666 M mannitol as in response to an isoosmotic strength of salt, 0.333 M (average peak heights of 1.65 and 1.85 μM respectively; Figure 1a and b). In these and subsequent experiments, the traces presented are the means of five individual seedling responses. Both of these calcium transients were significantly inhibited by pretreatment with 10 mM La^{3+} (Figure 1a and b). A smaller, but significant inhibition of the calcium transient in response to mannitol (Figure 1c) and to salt (Figure 1d) was also observed after pretreatment with 10 mM EGTA (Figure 1c and d). The fact that both lanthanum and EGTA inhibit the salt-and mannitol-induced $[Ca^{2+}]_{cyt}$ elevations indicate the involvement of extracellular calcium in this response. However, neither of these inhibitors abolish the response totally, suggesting the additional involvement of intracellular calcium stores in the drought- and salt-induced $[Ca^{2+}]_{cyt}$ responses of *Arabidopsis*. We went on to investigate whether these $[Ca^{2+}]_{cyt}$ elevations could be correlated with an end response, and for this purpose, measured the expression of three genes known to be regulated by osmotic stress. We first established the experimental conditions with which to assay induction of expression of the

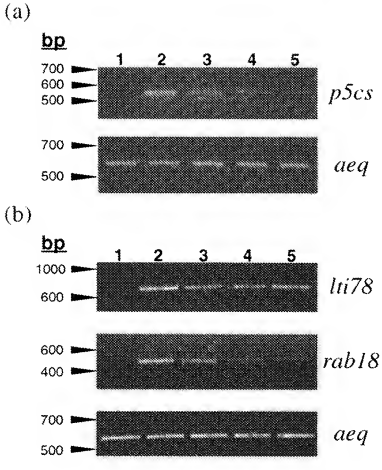

Figure 2. Calcium-dependent mannitol- and salt-induced gene expression in *Arabidopsis* seedlings.
(a) Ethidium bromide-stained agarose gel run to display DNA fragments produced by the specific RT-PCR amplification of *p5cs* and aequorin (control) mRNA transcripts. 7-day-old seedlings were treated for 4 h with (1) water, (2) 0.666 M mannitol, (3) 0.666 M mannitol after lanthanum pretreatment, (4) 0.333 M salt, (5) 0.333 M salt after lanthanum pretreatment.
(b) Ethidium bromide-stained agarose gel run to display DNA fragments produced by the specific RT-PCR amplification of *lti78*, *rab18* and aequorin, order as above.

At-P5CS (*p5cs*) gene in 7-day-old *Arabidopsis* seedlings, by measuring steady-state transcript levels. At 0.666 M mannitol *p5cs* gene expression reached maximal levels after 4 h exposure (data not shown). This time point was used thereafter for assessing the induction of the *p5cs* gene. For comparison, expression of *rab18* and *lti78* was assessed at this time point also. When comparing induction of *p5cs* expression by relatively high concentrations of salt (0.333 M) and an isoosmotic strength of mannitol (0.666 M) (Tsiantis *et al.*, 1996), lower levels of expression of the *p5cs* gene were observed in response to salt than to mannitol (Figure 2a). The RT-PCR data presented are representative of three separate experiments. RT-PCR of aequorin transcripts expressed constitutively in each sample was used as a control (Knight *et al.*, 1996). In order to gauge whether calcium was involved in the induction of the *p5cs* gene by salt and drought (mannitol), the calcium channel blocker lanthanum chloride was used. Preincubation in 10 mM lanthanum chloride for 30 min prior to stimulation significantly reduced the levels of *p5cs* expression induced in response to both salt and mannitol (Figure 2a). To assess whether or not this effect was specific to *p5cs* only, expression of two other genes, *rab18* and *lti78* was measured under the same conditions (Figure 2b). In the case of both *rab18* and *lti78*, mannitol induced a higher level of gene expression than did an isoosmolar concentration of salt, as was seen with *p5cs* expression. Mannitol-induced gene expression was strongly inhibited by preincubation in lanthanum chloride, although inhibition of the much lower salt-induced expression levels was not observed for either *rab18* or *lti78*. This contrasts with the results obtained with *p5cs* in which clear inhibition of salt-induced gene expression by lanthanum was seen (Figure 2a). The data indicated a requirement for calcium as a second messenger for the complete induction of these three genes at least in response to drought stress.

We investigated further the role of calcium in the mannitol-induction of one of these genes, *p5cs*, by the use of additional inhibitors of calcium channel activity and by external application of divalent cations (Figure 3a). A 30 min preincubation with 1 mM verapamil, a phenylalkylamine type calcium channel inhibitor which blocks predominantly L-type calcium channels (White, 1996), caused the most significant inhibition of *p5cs* gene expression in response to 0.666 M mannitol. Gadolinium (10 mM), which blocks stretch-activated plasma membrane calcium channels in animal cells (Yang and Sachs, 1989), and the calcium chelator EGTA caused smaller, but significant reductions

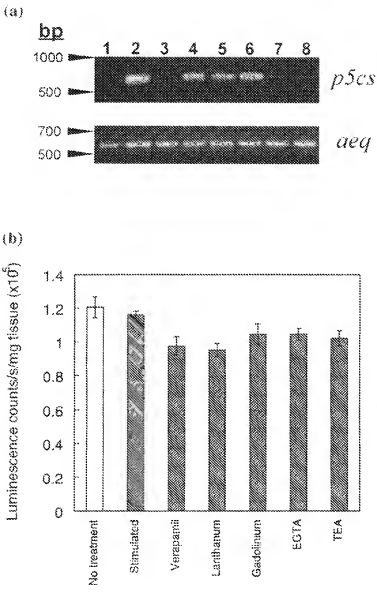

Figure 3. Effect of inhibitors and divalent cations on mannitol-induced *p5cs* gene expression
(a) Ethidium bromide-stained agarose gel run to display DNA fragments produced by the specific RT-PCR amplification of *p5cs* and aequorin mRNA transcripts. 7-day-old seedlings were treated for 4 h with either water (1) or 0.666 M mannitol (2 to 6), 100 mM calcium chloride (7) or 100 mM magnesium chloride (8). Sample (3) was pretreated with verapamil, (4) with gadolinium, (5) with EGTA and (6) with TEA.
(b) Relative levels of ATP after inhibitor and mannitol treatment of *Arabidopsis* seedlings. An ATP-dependent luminescence assay was used to determine the ATP levels in 7-day-old seedlings treated for 4 h with water (no treatment), 0.666 M mannitol (stimulated) or 0.666 M mannitol after pretreatment with verapamil, lanthanum, gadolinium, EGTA or TEA. Results are expressed as luminescence counts sec^{-1} mg^{-1} tissue.

in the amount of mannitol-induced *p5cs* gene expression. As a control the potassium channel blocker TEA (Fan *et al.*, 1994) was used. TEA caused negligible effects on the mannitol-induced level of *p5cs* gene expression. Calcium chloride (at 100 mM) and magnesium chloride (also at 100 mM), in the absence of mannitol, both caused a slight induction of *p5cs* gene expression. This suggests that this was probably due to the osmotic effect of adding these salts, and that calcium alone is not sufficient for induction of *p5cs*. To ascertain that these inhibitors did not mediate their effect simply by causing general toxic effects, we measured the energy status of the plants by determining ATP levels after inhibitor/mannitol treatments. In all cases, ATP levels measured by a luciferase assay were similar to those of controls (Figure 3b). All values represent the mean of data recorded from three separate samples. Treatment with 0.666 M mannitol only for 4 h had no significant effect on ATP levels (Figure 3b). EGTA and gadolinium pretreatment both caused a small reduction (10%) in ATP levels compared with treatment by mannitol alone. A slightly greater inhibition of ATP levels was seen after pretreatment with lanthanum (18%) or verapamil (16%). These levels of reduction in ATP levels were comparable with those seen after treatment with TEA (12%), an inhibitor having little effect on *p5cs* expression. The degree of ATP depletion seen after treatment with lanthanum, EGTA, gadolinium and verapamil does not appear to be large enough to account for the relatively strong inhibition of *p5cs* gene expression seen with these inhibitors. It was concluded therefore, that the observed effects of lanthanum, gadolinium, verapamil, and EGTA on *p5cs* gene expression were due to *bona fide* effects on Ca^{2+} homeo-

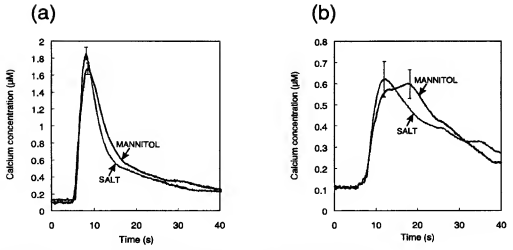

Figure 4. $[Ca^{2+}]_{cyt}$ responses to high and low concentrations of salt and mannitol in *Arabidopsis* seedlings.
(a) High salt (0.333 M; half-tone) and high mannitol (0.666 M; full-tone) in plants expressing cytosolic aequorin.
(b) Low salt (0.166 M; half-tone) and low mannitol (0.333 M; full-tone) in plants expressing cytosolic aequorin. Salt or mannitol was added 5 sec after the beginning of the trace. Traces shown are averages of five individual measurements and error bars indicate standard error of the mean.

stasis. Together, these $[Ca^{2+}]_{cyt}$ measurements and gene expression data suggested a role for intracellular calcium in the signalling pathways leading to the induction of at least three genes (*p5cs*, *lti78* and *rab18*) in response to drought and at least one in response to salinity stress (*p5cs*), and that influx of external calcium plays an important part in these events. Additionally, the fact that *p5cs* gene expression and both salt- and drought-induced $[Ca^{2+}]_{cyt}$ responses are only partially inhibited by chelating extracellular calcium with EGTA suggested that internal calcium stores may also be required for full induction of gene expression. This possibility was investigated subsequently (see below).

We attempted to discover whether the discrimination between drought and salinity stresses which is known to occur in plants (Tsiantis *et al.*, 1996) might be mediated via calcium signalling in *Arabidopsis*. At relatively high concentrations of mannitol and salt, only a small difference was seen between the average peak heights of the $[Ca^{2+}]_{cyt}$ elevation, salt causing a slightly higher peak than mannitol (1.85 μM and 1.65 μM respectively; Figure 4a). To ascertain that this similarity was not due to the proximity to some kind of signal-intensity threshold, the experiments were repeated at lower concentrations of salt and mannitol. With isoosmotic concentrations of 0.166 M salt and 0.333 M mannitol the response was more variable than at the higher concentration, but still quite similar between salt and mannitol, with an average peak height of approximately 0.6 μM for both treatments (Figure 4b). Levels of mannitol-induced *p5cs* gene expression were, however, markedly greater than those induced by isoosmolar concentrations of salt, at both high and low concentrations (Figure 5). Therefore, there was no obvious correlation between the amount of *p5cs* gene expression and the magnitude of the calcium transients measured. At the relatively high concentrations, there was similarly no correlation of $[Ca^{2+}]_{cyt}$ transient magnitude with levels of *rab18* and *lti78* expression (Figure 2b). These data indicated that some factor other than calcium must be involved in the transduction of and discrimination between salinity and drought signals in *Arabidopsis*. It must be pointed out that discrimination between these two signals could occur via long-term signal transduction events rather than the short-term $[Ca^{2+}]_{cyt}$ elevation.

The partial inhibition of both the $[Ca^{2+}]_{cyt}$ elevation and the level of *p5cs* gene expression by chelating external calcium with EGTA suggests that external calcium influx does not account for the whole response, and that intracellular calcium stores may also be involved. Of the possible organelles which could be releasing calcium during drought stress, the vacuole seems particularly likely. Previous studies have suggested a role for the vacuole in osmotic stress signalling (Allen and Sanders, 1994a). In order to ascertain whether or not vacuolar calcium release contributed to the mannitol-induced $[Ca^{2+}]_{cyt}$ elevation in *Arabidopsis*, we compared the calcium transient in response to 0.666 M mannitol in plants expressing aequorin in the cytosol and plants expressing aequorin in the cytosolic microdomain adjacent to the vacuolar membrane (vacuolar microdomain) (Knight *et al.*, 1996). After the addition of 0.666 M mannitol, the $[Ca^{2+}]_{cyt}$ rose to similar levels in the cytosol (1.5 μM) as in the vacuolar microdomain (1.4 μM), the peak being slightly more prolonged in the vacuolar microdomain (Figure 6a). Because not all the aequorin is targeted to the tonoplast membrane (Knight *et al.*, 1996), absolute calibrations of $[Ca^{2+}]_{cyt}$ in the microdomain cannot be made. Thus direct comparison of absolute $[Ca^{2+}]_{cyt}$ values between total cytosolic and vacuolar

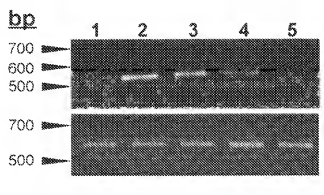

Figure 5. Differing levels of *p5cs* transcript accumulation upon isoosmotic salt and mannitol treatments in *Arabidopsis* seedlings. Ethidium bromide-stained agarosa gel run to display DNA fragments produced by the specific RT-PCR amplification of *p5cs* and aequorin mRNA transcripts. 7-day-old seedlings were treated for 4 h with (1) water, (2) 0.666 M mannitol, (3) 0.333 M mannitol, (4) 0.333 M salt, (5) 0.166 M salt.

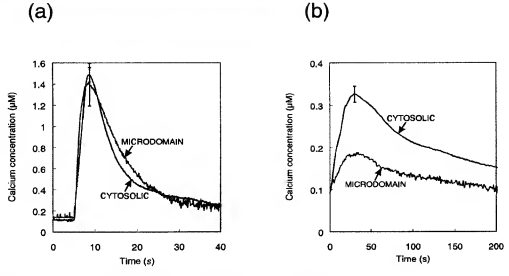

Figure 6. Changes in $[Ca^{2+}]_{cyt}$ in the cytosol and vacuolar microdomain of *Arabidopsis* seedlings responding to mannitol.
(a) Effect of mannitol on cytosolic (full-tone) and microdomain (half-tone) $[Ca^{2+}]_{cyt}$.
(b) Effect of adding 10 mM hydrogen peroxide on cytosolic (full-tone) and microdomain (half-tone) $[Ca^{2+}]_{cyt}$. Mannitol was added 5 sec after the beginning of the trace and hydrogen peroxide immediately at the beginning of the trace. Traces shown are averages of five individual measurements and error bars indicate standard error of the mean.

microdomain aequorin plants are not possible. However, relative comparisons between magnitude and duration of $[Ca^{2+}]_{cyt}$ responses to different stimuli can be made to give an indication of the involvement of the vacuole in calcium signalling. As such, the similarity of average $[Ca^{2+}]_{cyt}$ peak height of the measurements made in the two sets of plants is unique and different to any other response we have studied. Previously, we found the peak height of the vacuolar microdomain Ca^{2+} response to cold was in the region of 70% of that seen in the cytosol (Knight *et al.*, 1996). Similarly, the response to 10 mM hydrogen peroxide is significantly lower in the vacuolar microdomain than in the cytosol (Figure 6b). The unusually large vacuolar microdomain calcium peak height in response to mannitol suggests a significant contribution to the calcium signal is due to release from the vacuole. The fact that the initial rate of calcium elevation is also faster in the microdomain plants than the cytosolic plants (time taken for doubling of calcium concentration 0.97 sec versus 0.59 sec) seems to substantiate the suggestion of a vacuolar release of calcium in response to mannitol. This does not of course exclude the additional possible involvement of other intracellular calcium stores, e.g. the ER, in the response of *Arabidopsis* to salt and drought.

As the vacuole seemed to be involved in the $[Ca^{2+}]_{cyt}$ response to mannitol in *Arabidopsis* and the most likely site of perception for this signal is the plasma membrane, we were interested in understanding how this perception might be communicated to the vacuole. One way in which calcium can be released from the vacuole is through IP_3-sensitive channels (Alexandre *et al.*, 1990; Allen *et al.*, 1995). To determine whether the mannitol-induced $[Ca^{2+}]_{cyt}$ elevation occurs as a result of IP_3-mediated calcium signalling, plants expressing vacuolar microdomain aequorin were pretreated with the inhibitors neomycin sulphate, U-73122 and lithium chloride, each of which inhibits IP_3 signalling. Neomycin inhibits phospholipase C, thus reducing the amount of IP_3 released (Nakashima *et al.*, 1987; Atkinson *et al.*, 1993). Pretreatment in 50 μM neomycin for 30 min resulted in a slower vacuolar microdomain calcium

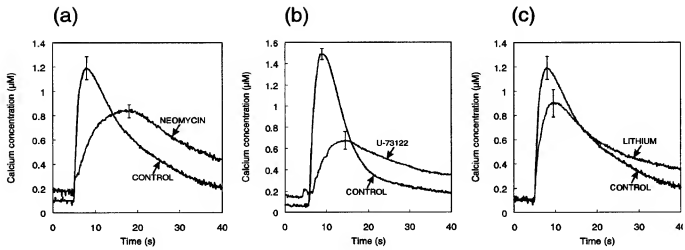

Figure 7. Effect of inhibitors of inositol signalling on mannitol $[Ca^{2+}]_{cyt}$ responses measured in the vacuolar microdomain.
(a) Untreated (full-tone) versus neomycin pretreated (half-tone) microdomain plants.
(b) Untreated (full-tone) versus U-73122 pretreated (half-tone) microdomain plants.
(c) Untreated (full-tone) versus lithium chloride pretreated (half-tone) microdomain.
Mannitol was added 5 sec after the beginning of the trace. Traces shown are averages of five individual measurements and error bars indicate standard error of the mean.

response to mannitol and a reduction in average peak height from 1.2 μM to 0.8 μM (Figure 7a). The response declined more slowly in neomycin pretreated plants than in non-pretreated plants. U-73122 is also an inhibitor of phospholipase C and blocks G-protein activation of phospholipase C (Yule and Williams, 1992). A 30 min pretreatment with 50 μM U-73122 caused a dramatic reduction in the response to 0.666 M mannitol from an average peak height of 1.45 μM to 0.65 μM (Figure 7b). Lithium chloride interrupts phosphoinositide cycling by inhibiting *myo*-inositol-1-phosphatase (IMP) (Berridge and Irvine, 1984; Loewus and Loewus, 1982), the enzyme which dephosphorylates inositol 1-phosphate to yield free inositol, the substrate required for *de novo* production of IP_3 (Loewus and Loewus, 1983; Gillaspy *et al.*, 1995). Lithium affects a number of processes in animals by its inhibition of IMP. For instance it disrupts axis determination in *Xenopus* and inhibits the increase in IP_3 levels which occurs during normal mesoderm induction (Maslanski *et al.*, 1992). Effects such as these can be counteracted by cointroduction of inositol with the lithium, indicating that the altered signalling seen is attributable to inositol depletion (Berridge *et al.*, 1989). Cloning of the *IMP* gene from tomato (*LelMP*), has confirmed, contrary to previous doubts, that plant IMP also is sensitive to lithium, and therefore the cation can be used as an inhibitor of IP_3 levels in plants (Gillaspy *et al.*, 1995). In our experiments, 20 mM lithium chloride caused a significant reduction in the peak height of the Ca^{2+} response to 0.666 M mannitol at the vacuolar membrane, from an average peak height of 1.2 μM to 0.9 μM (Figure 7c). This suggests that the mannitol-induced calcium elevation is in part due to an IP_3-mediated release of intracellular calcium which can be inhibited by inositol depletion.

Discussion

We have demonstrated in intact whole plants that both salt and drought stresses cause a rapid increase in $[Ca^{2+}]_{cyt}$ levels in *Arabidopsis*, as reported previously in response to salt in maize root protoplasts (Lynch *et al.*, 1989). The calcium channel blocker lanthanum chloride caused a dramatic reduction in the magnitude of the $[Ca^{2+}]_{cyt}$ elevation in response to mannitol or salt (Figure 1). Although lanthanum acts mainly as an extracellular calcium channel blocker (Knight *et al.*, 1996), it is possible some blocking of intracellular calcium channels occurred due to entry of lanthanides into cells (Allen and Sanders, 1994b; Gelli and Blumwald, 1993; Klüsener *et al.*, 1995; Quiquampoix *et al.*, 1990), therefore the result should be interpreted with some caution. However, a smaller but significant inhibition of the response was also seen after preincubation with EGTA, indicating that an influx of extracellular calcium constitutes at least a significant part of the observed $[Ca^{2+}]_{cyt}$ elevation.

Expression of the gene *p5cs*, which encodes a protein with a known protective role in the response to drought, was clearly induced by relatively high concentrations of salt and mannitol, though less so by salt than by isoosmolar concentrations of mannitol (Figure 2a). In the case of both salt and mannitol, induction was inhibited by lanthanum, suggesting that the initial $[Ca^{2+}]_{cyt}$ elevation may be correlated with the full induction of the gene. Similar experiments measuring expression of the genes *lti78* and *rab18*, which encode proteins of unknown function, suggested that other drought-responsive genes were also dependent on calcium for complete induction. The lack of a clear inhibition of the salt-induced *lti78* and *rab18* gene expression may reflect the low levels of expression measured, as much as they

indicate a lack of dependency on Ca^{2+} for salt-induced expression. Further characterisation of the role of Ca^{2+} in *p5cs* induction revealed that EGTA caused some inhibition of expression, consistent with the observed inhibition of the $(Ca^{2+})_{cyt}$ elevation, and that gadolinium was also capable of inhibiting *p5cs* expression (Figure 3a). Gadolinium blocks stretch-activated Ca^{2+} channels in animal cells (Yang and Sachs, 1989) so this result is in accordance with what would be expected in response to a stress which involves changes in cell turgor and therefore presumably mechanical stress. In bacteria, gadolinium inhibits both stretch-activated channel activity and the loss of ions and metabolites from the cell which usually results from hypoosmotic shock (Berrier *et al.*, 1992). This evidence has implicated stretch-activated channels in bacterial turgor sensing and it has been suggested that as stretch-activated Ca^{2+} channels have been demonstrated in plants (Cosgrove and Hedrich, 1991), that a similar mechanism may operate in plants (Lew, 1996). The dramatic effect of verapamil on *p5cs* gene expression suggests the substantial involvement of L-type Ca^{2+} channels. It could be argued that the inhibitors used in these experiments had general toxic effects and that the inhibition of mannitol-induced gene expression observed was simply as a result of general failure to efficiently transcribe genes under these conditions. To provide evidence that these inhibitors were acting specifically through their effects on Ca^{2+} homeostasis, ATP levels were measured in treated and non-treated plants in order to gauge energy status. In the case of lanthanum, EGTA, gadolinium and verapamil, ATP levels were not reduced to below 83% of the controls, and this small difference does not appear to be sufficient to account for the marked degree of inhibition of *p5cs* expression by these inhibitors. Furthermore, these levels were similar to those seen after TEA treatment (88%): an inhibitor with little effect on *p5cs* gene expression. None of these inhibitors showed any effect on the levels of the control (aequorin) transcripts, also suggesting that general inhibition of transcription was not occurring. It should be also borne in mind that inhibitors which disrupt drought-induced calcium signalling events are likely to inhibit a number of downstream responses which are normally required for the amelioration of drought stress. It is thus quite possible that the additional burden of surviving drought stress without the availability of these normal mechanisms would impose an energy cost on the cells involved and thus lead to reduced ATP levels. Overall, it can be concluded that the inhibition of *p5cs* gene expression by lanthanum, gadolinium, verapamil and EGTA was for the most part, or entirely, due to their effects on calcium signalling. It seems that the potassium channel blocker TEA has little effect on mannitol-induced *p5cs* gene expression. In *E. coli* K^+ is the primary osmolyte accumulating in response to hyperosmotic pressure and it has been suggested it may act as a regulatory signal during osmotic stress (Higgins *et al.*, 1987). Also, the bacterial transport systems for K^+ uptake are regulated by osmotic stress (Higgins *et al.*, 1987), indicating a role for K^+ in the prokaryote osmotic stress response. In plants too there is a possible role for K^+, as potassium uptake is an integral part of the salt tolerance mechanism in glycophytes (Wu *et al.*, 1996). Therefore there may be some involvement of potassium channels in the mediation of responses to drought stress in plants. However, this does not seem to include the induction of *p5cs* gene expression in *Arabidopsis*.

The small induction of *p5cs* expression caused by adding 100 mM $CaCl_2$ (a treatment which we have shown causes a $[Ca^{2+}]_{cyt}$ transient reaching c.a. 1 μM; Knight, Trewavas and Knight, unpublished) was equalled by a similar level of induction by 100 mM $MgCl_2$. This, therefore, cannot be a calcium specific effect and is more likely to be due simply to the osmotic effect of adding these salts. These data indicate the requirement for a signalling factor(s) in addition to calcium for full induction of *p5cs* gene expression.

We compared the $[Ca^{2+}]_{cyt}$ transients and levels of *p5cs* gene expression induced by isoosmolar concentrations of mannitol and salt to see if Ca^{2+} might be involved in the discrimination between these two signals. A number of osmotically induced genes have been shown to be expressed preferentially in response to either drought or salt stress in wheat, tomato and *Mesembryanthemum crystallinum* (Erdei *et al.*, 1990; Galiba *et al.*, 1993; Chen and Tabaeizadeh, 1992; Tsiantis *et al.*, 1996), indicating that different pathways may be involved in the transduction of these two stresses in these particular species. We have shown that isoosmotic concentrations of salt and mannitol induce notably different levels of expression of the *p5cs*, *rab18* and *lti78* gene after 4 h exposure in 7-day-old seedlings of *Arabidopsis* (Figures 2 and 5). This difference is seen for *p5cs* both at relatively high and low concentrations of salt and mannitol (Figure 5). The small difference between the $[Ca^{2+}]_{cyt}$ elevations observed in response to either mannitol or salt at relatively high or relatively low concentrations (Figure 4) suggests that the ionic effects of salt-stress have little involvement in the increase in $[Ca^{2+}]_{cyt}$, rather that the elevation is mostly attributable to the osmotic stress caused by both treatments. It also indicates that calcium signalling is not used as a mechanism for distinguishing between the two stresses in *Arabidopsis*. Taken together, these data (Figures 4 and 5) also suggest that calcium is necessary but not sufficient for drought and salt induction of *p5cs*, a conclusion supported by other data (above) showing that the addition of external Ca^{2+} did not specifically induce gene expression (Figure 3a). The data suggest that the discrimination between mannitol and salt in *Arabidopsis* is mediated by a calcium-independent signalling factor(s). $[Ca^{2+}]_{cyt}$ measurement data obtained at lower concentrations of salt

and mannitol were more variable than those obtained at the higher concentrations (Figure 4b), indicating that perhaps these concentrations were close to some type of a response threshold. The differences between the effects of salt and mannitol may be due to the fact that mannitol cannot enter the cell whereas sodium and chloride ions may be transported into the cell and transported into the vacuole, thus reducing the potentially high osmotic difference between cytosol and apoplast. It is also possible that ABA, which has been implicated in the transduction of drought signals (for review, see Skriver and Mundy, 1990) may be less effectively mobilised by sodium chloride treatment, or that its action is in some way negated by salination. However, the difference in salt-and mannitol-induction of gene expression is also observed to a similar degree with both the *lti78* and *rab18* genes, one of which is ABA-regulated and the other which is not. It seems unlikely, therefore, that ABA is the discriminating factor in this case.

The vacuole has previously been implicated in the perception of osmotic stress (Alexandre and Lassalles, 1991) and it has already been shown that osmotic stress enhances the competence of *Beta vulgaris* IP_3-sensitive vacuolar calcium channels to respond to IP_3 (Allen and Sanders, 1994a). These data, taken together with consideration of the size of the vacuole and its role in mediating the cellular response to turgor make vacuolar calcium a strong candidate for organelle-specific signalling in response to osmotic stress. The incomplete inhibition of the drought- and salt-induced $[Ca^{2+}]_{cyt}$ elevation by lanthanum or EGTA (above), which should exert their effects mainly on external calcium influx (Knight *et al.*, 1996) suggested that an internal store may also contribute to the $[Ca^{2+}]_{cyt}$ elevation. In order to gauge whether the vacuole might be the internal store involved, we compared the calcium dynamics occurring around the vacuole (vacuolar microdomain; Knight *et al.*, 1996) in response to osmotic stress with those occurring throughout the cytosol. Using *Arabidopsis* expressing apoaequorin targeted to the cytosolic face of the vacuolar membrane (Knight *et al.*, 1996), we discovered that the calibrated $[Ca^{2+}]_{cyt}$ values at the microdomain were equal to or higher than those occurring in the cytosol, were of a slightly longer duration and exhibited a faster initial rate (Figure 6a). This was particularly noteworthy as we have compared the effect of a number of other stimuli on microdomain and cytosolic calcium elevations, and in every other case the vacuolar microdomain signal peak height is considerably lower than in the cytosol. This is seen in response to touch (Knight and Knight, unpublished), cold (Knight *et al.*, 1996) and hydrogen peroxide (Figure 6b). These facts along with the faster and more prolonged kinetics of the microdomain response suggest that there is a considerable involvement of the vacuole in the salt/drought stress response. The observation that the vacuole may be involved in releasing calcium led us to investigate the possible role for phosphoinositide lipid signalling. Previous work with CTC staining in corn root protoplasts (Lynch and Läuchli, 1988) indicated that salt-induced depletion of intracellular calcium stores is mediated by activation of the phosphoinositide system. The effect of the inhibitors of phospholipase C (neomycin and U-71322), and *myo*-inositol-1-phosphatase (lithium) on reducing the elevation in $[Ca^{2+}]_{cyt}$ gives strong support for the suggestion that the vacuolar calcium release occurs via phosphoinositide signalling. The prolongation of the mannitol-induced elevation of $[Ca^{2+}]_{cyt}$ by lithium, neomycin or U-73122 may be due to a reduced rate of inositol phosphate recycling.

In summary, the work described in this paper demonstrates the key role played by the second messenger calcium in mediating salt- and drought-induced gene expression and the role of the vacuole in this response. Understanding the signal transduction pathway leading to the expression of proteins with protective functions, will be important in the future. Once more components regulating the proline biosynthetic pathway and other pathways leading to the production of osmoprotectants are identified and manipulated it might be possible to engineer plants with enhanced abilities to respond quicker or better to salt and drought stress (Vierling and Kimpel, 1992). It will be particularly interesting to identify the component(s) responsible for discrimination between salt and drought. Salt and drought calcium signalling mutants of *Arabidopsis* will also be useful tools for understanding the role for calcium in other aspects of salt and drought physiology.

Experimental procedures

Measurement of $[Ca^{2+}]_{cyt}$ by aequorin luminometry

Transgenic *Arabidopsis thaliana* (a kind gift from Janet Braam and Diana H. Polisensky, Rice University, TX, USA; Polisensky and Braam, 1996) plants expressing cytosolic apoaequorin were used for $[Ca^{2+}]_{cyt}$ measurements. Plants expressing an apoaequorin-pyrophosphatase fusion protein (Knight *et al.*, 1996) were used for the measurement of $[Ca^{2+}]_{cyt}$ adjacent to the vacuole (the vacuolar microdomain). Seedlings were grown on full strength MS medium (Murashige and Skoog, 1962), 0.8% agar at 21°C with a 16 h photoperiod and were used when 6–7 days old. Reconstitution of aequorin was performed *in vivo* essentially as described previously (Knight *et al.*, 1991) by floating seedlings on water containing 2.5 μM coelenterazine in the dark, overnight at 21°C.

Experiments were performed by placing individual seedlings in a transparent plastic cuvette containing 0.2 ml water. The cuvette was placed inside a digital chemiluminometer with a discriminator as previously described (Campbell, 1988; Knight and Knight, 1995). Luminescence counts were integrated every 0.1 sec. After 5 sec of counting, 0.4 ml mannitol or sodium chloride solution was injected into the cuvette via a light-tight 1 ml syringe inserted into a light-tight port in the luminometer sample-housing, to give a

final concentration of 0.166 M or 0.333 M sodium chloride, and 0.333 M or 0.666 M mannitol (both Analar grade, BDH, Poole, UK). The volume of liquid added and a slow rate of injection were determined empirically as being optimum for giving a negligible touch-induced $[Ca^{2+}]_{cyt}$ elevation in the seedlings (data not shown). Plants were preincubated for 30 minutes in 10 mM lanthanum chloride, 10 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 50 μM neomycin sulphate, 20 mM lithium chloride (all Sigma, Poole, UK), 50 μM 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122; Alexis Corporation, Nottingham, UK) or water when required. After 30 min, seedlings were removed from the inhibitor and placed in the luminometer cuvette containing water as described above. At the end of each experiment the remaining aequorin was discharged by the addition of an equal volume of 2 M $CaCl_2$ 20% ethanol. Luminescence values were calibrated as calcium concentrations as described previously (Knight *et al.*, 1996).

Measurement of salt- and mannitol-induced p5cs, rab18 *and* Iti78 *gene expression using reverse-transcriptase-PCR*

Oligonucleotide primers were designed to amplify part of the coding sequence of the *Arabidopsis p5cs* transcript (Savouré *et al.*, 1995), a single gene in the *Arabidopsis* genome, to give a 500 bp PCR product from a cDNA template which corresponded to a region of the gene spanning 5 introns. Oligonucleotide primers were also designed to amplify parts of the coding sequences of the *Arabidopsis* transcripts *Iti78* and *rab18* (Nordin *et al.*, 1993; Lång and Palva 1992) to give PCR products of 778 bp and 416 bp respectively from cDNA template. The *Iti78* and *rab18* PCR products corresponded to a region of these genes spanning one intron each.

Oligonucleotide primers were obtained from Genosys (Cambridge, UK) and Cruachem (Glasgow, UK) and their sequences (5' to 3') were as follows:

Forwardp5cs: 5'(GGAGGAGCTAGATCGTTCAC)3';
Reversep5cs: 5'(TCAGTTCCAACGCCAGTAGA)3';
ForwardIti78: 5'(TCTTCCGATTACACCAAACC)3';
Reverselti78: 5'(CTGATTCACTACCAAAGCCC)3';
Forwardrab18: 5'(CGATCCAGCAGCAGTATGAC)3';
Reverserab18: 5'(TTCGAAGCTTAACGGCCACC)3'.

Approximately 20–25 mg of 7-day -old *Arabidopsis* seedlings expressing cytosolic aequorin (grown as described above) were placed in water or 10 mM lanthanum chloride for 30 min, after which they were placed in the appropriate salt (0.166 M, 0.333 M), mannitol (0.333 M, 0.666 M), calcium chloride (100 mM) or magnesium chloride (100 mM) solution or water. Seedlings were also incubated in 10 mM EGTA, 10 mM gadolinium chloride, 10 mM tetraethylammonium chloride (TEA) or 1 mM verapamil hydrochloride (all from SIGMA, Poole, UK) before incubation in 0.666 M mannitol solution. Seedlings were harvested after 4 h, this having been previously determined as the time at which expression of the *p5cs* gene was maximal (data not shown). Total RNA was prepared from seedling tissue using RNeasy plant RNA minipreps (Qiagen, Dorking, UK) and cDNA synthesised. For each reaction 1 μg total RNA was used and 1 μl oOligo(dT) (500 μg ml^{-1}) added in a total volume of 12 μl. The mixture was heated to 70°C for 10 min and quick chilled on ice, to denature RNA. To this was added, 4 μl 5× first strand buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl 0.1 M DTT, 1 μl 10 mM dNTPs, 1 μl (200 units) Superscript II (RNaAse H^- reverse transcriptase, Life Technologies, Paisley, UK). cDNA synthesis was performed by incubating at 42°C for 50 minutes, and the enzyme inactivated at 70°C for 15 minutes. The cDNA was diluted for use in the PCR reaction; 5 μl of 1:1000 dilution with the specific *p5cs* primers (see above), 10 μl of 1:1000 dilution with the specific *rab18* primers or 10 μl of 1:100 dilution with the specific *Iti78* primers, in a total of 50 μl. The cDNA was amplified under the following conditions: 94°C for 5 min, 60°C for 5 min, 72°C for 5 min; followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and ending with 15 min at 72°C. Control PCR was performed using 5 μl of a 1:10 000 dilution of the cDNA and previously made primers to the aequorin gene (Knight *et al.*, 1996) under the same conditions. PCR products were loaded and run on 1% agarose TBE gels (10 μl of reaction for *p5cs, Iti78* and *rab18* and 5 μl for aequorin) and visualized by ethidium bromide fluorescence.

Measurement of ATP levels

Seedlings were incubated in either inhibitor solutions or water for 30 min, after which time they were transferred to 0.666 M mannitol, or water for 4 h. Three replicates of 10 seedlings each (c.a. 7.5 mg fresh weight per sample) were harvested and frozen in liquid nitrogen. The frozen tissue was first homoganized alone, then 200 μl of 25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane *N,N,N',N'*-tetraacetic acid, 10% glycerol (v/v), 1% Triton X-100 (v/v) was added and the tissue was homogenized again. Homogenates were centrifuged for 5 min at 13 000 ***g*** and the supernatants kept for analysis. To a luminometer cuvette, 20 μl of supernatant was added and background luminescence counts were determined. For the assay, 100 μl luciferase solution (10 mg ml^{-1} in glycine buffer, Sigma, Dorset, UK) was added and mixed, and luminescence counts measured as 5 sec integrations after a delay of 5 sec. Controls in which 100 μl luciferase solution was added to 20 μl of homogenisation buffer only, were used to subtract background levels of luminescence. Dilutions of homogenates were tested over three orders of magnitude to ensure that the assay was linear.

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